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Recent Advances in Noninvasive Techniques to Monitor Hormone-Behavior Interactions

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ABSTRACT This paper reviews recent advances in field endocrinology, a focus as well as a method in primatology and behavioral ecology that permits the examination of social behavior and life history through hormonal investigations in natural settings. Endocrine data complements the traditional behavioral data collected by field scientists by providing quantitative measures for the examination of adaptive tradeoffs, costs of social strategies, and reproductive and social significance of mating events. Further, investigations of the physiological mechanisms of reproductive constraint provide tests of the adaptive significance of reproductive skew in cooperative and competitive breeders. Hormone data also can provide insights into the costs of competition and aggression and the role of temperament in individual reproductive success and the evolution of social systems. New, noninvasive methods for the collection of this information have augmented and expanded field endocrinology through the use of techniques that do not require potentially confounding physical or physiological manipulations. Specifically, urine and fecal samples can be collected from free-ranging animals and contain gonadal and adrenal hormones that parallel profiles of serum hormones. Sampling, preservation, extraction, and assay methods for the analysis of excreted steroids are reviewed along with the species and questions to which these methods have been applied. Yrbk Phys Anthropol 41:1–23, 1998.

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Field or evolutionary endocrinology, a field which addresses the observation that vertebrate species often differ considerably in endocrine regulation as well as behavior and ecology, unites laboratory- and field-based scientific methods. Currently, field endocrinology is undergoing a period of rapid growth due to the development of noninvasive techniques for monitoring physiological functions. The field endocrinologist can examine hormone-behavior relationships through the study of animals in their natural habitats and social settings. Greater attention to physiological processes is yielding new information about evolutionary processes and adding new rigor to the comparative method (Finch and Rose, 1995).

Investigation of the role of hormones, however, requires attention to the normal ecological and social context of endocrine function and its relation to behavioral variation, life history, and Darwinian fitness. Not surprisingly, field studies have revealed that both absolute levels and the temporal patterning of hormones under natural conditions may differ substantially from that seen in captivity (Wingfield and Moore, 1987). The significance of these differences has been investigated using several different methods: ethological analyses comparing hormone levels before and after social interactions and experimental elicitation of hormonal responses using simulated social and environmental stimuli and manipulations (phenotypic engineering: Ketterson and Nolan, 1992) in which hormone levels are altered to investigate the fitness consequences of related suites of behaviors. Most of this research, focusing on reproductive strategies, was carried out initially using blood samples obtained by darting or trapping feral animals, primarily birds, reptiles, and small mammals (reviewed in Bronson, 1989; Wingfield et al., 1990; Moore, 1991), with some notable primate exceptions (baboons: Sapolsky, 1982, 1993; Phillips-Conroy et al., 1992; rhesus macaques: Bercovitch, 1992; Higley et al., 1992; Kaplan et al., 1995). Today, the development and validation of new, noninvasive endocrine methods using excreted steroids have broadened the application of this research to a wide variety of mammalian species (see Table 1). This paper will review these methods and their application to evolutionary questions in behavioral ecology and primatology.

BACKGROUND

From an evolutionary standpoint, hormones are of interest because they regulate reproduction, development, and the expression of behavior. A wide variety of endocrine factors has been linked to ecological and social variation, including gonadal and adrenal steroids, pituitary peptides, growth factors, and neurotransmitters. Assessing the status and responses of these factors presents a number of measurement challenges, especially in field settings. Steroids and pituitary peptides can be measured in serum, plasma, urine, saliva, or, in the case of steroids, fecal samples, while neurotransmitters (but not all neurotransmitter metabolites) must be measured in cerebrospinal fluid. Sampling blood or cerebrospinal fluid requires darting or trapping and anesthetization, whereas urine and feces often can be collected without disrupting animal activities.

TABLE 1. Validations of excreted steroids and steroid metabolites as measures of ovarian function and pregnancy in primates[1]

Taxa	Common name	Species	Fecal/urine steroid metabolites	Application	Reference
Indriidae	Verreaux's sifaka	*Propithecus verreauxi*	P_4, E_2	Ovarian cycle, ovulation, pregnancy	Brockman, 1994; Brockman et al., 1995; Brockman and Whitten, 1996
Callitrichidae	Cotton top tamarin, saddleback tamarin	*Saguinus oedipus*, *S. fuscicollis*	P_4, PdG, E_1, E_2	Ovarian cycle	Heistermann et al., 1993; Ziegler et al., 1996
	Common marmoset	*Callithrix jacchus*	P_4, PdG, E_1, E_2	Ovarian cycle, pregnancy	Heistermann et al., 1993; Ziegler et al., 1996
	Goeldi's monkey	*Callimico goeldii*	Estrogen	Ovarian cycle, ovulation	Pryce et al., 1994
Cebidae	White-fronted saki monkey	*Pithecia pithecia*	PdG, E_1C	Ovarian cycle, pregnancy	Shideler et al., 1994
	Black howler	*Alouatta palliatta*	E_2	Ovarian cycle, pregnancy	Clarke et al., 1991; Zucker et al., 1994, 1995
	Muriqui	*Brachyteles arachnoides*	E_2, P_4	Ovarian cycle, ovulation, pregnancy	Strier and Ziegler, 1994, 1997
Colobinae	Hanuman langur	*Trachypithecus entellus*	E_2, P_4, PdG, 20α-OHP	Ovarian cycle, pregnancy	Heisterman et al., 1995
Cercopithecinae	Longtailed macaque	*Macaca fascicularis*	E_1C, PdG	Ovarian cycle	Risler et al., 1987; Shideler et al., 1993
	Rhesus macaque	*Macaca mulatta*	E_2, P_4	Ovarian cycle	Stavisky 1994; Stavisky et al., 1995
	Baboon	*Papio cynocephalus*	E_2, P_4	Ovarian cycle, pregnancy	Wasser et al., 1991; Stavisky et al., 1995; Wasser, 1996
	Sooty mangabey	*Cercocebus torquatus*	E_2, P_4	Ovarian cycle, ovulation	Whitten and Russell, 1996; Whitten, 1997
Hominoidea	Bonobo	*Pan paniscus*	E_1C, P_4	Ovarian cycle, pregnancy	Heisterman et al., 1996
	Orangutan	*Pongo pygmaeus*	E_1C, HCG, LH	Ovarian cycle	Knott, 1993, 1996, 1997
	Chimpanzee	*Pan troglodytes*	E_1C	Ovarian cycle	Knott, 1993
	Human	*Homo sapiens*	E_2, T	Sex determination of paleofeces	Sobolik et al., 1996

[1] Abbreviations used: E_1, estrone; E_1 C, estrone conjugates; E_2, estradiol; 20α-OHP, 20 alpha-hydroxy progesterone; T, testosterone; P_4, progesterone; PdG, pregnanediol glucuronide; HCG, human choronic gonadotropin; LH, lutenizing hormone.

Each sampling regimen represents a different slice of physiological events (see Fig. 1). Concentrations of neurotransmitters or their metabolites in cerebrospinal fluid (CSF) reflect the activity of specific types of neurons but are nonspecific in regard to the neuroanatomical locale of that activity. Moreover, CSF concentrations of neurotransmitter metabolites like 5-hydroxyindole acetic acid (5-HIAA), the breakdown product of serotonin, reflect transmitter metabolism as well as release. Serum and plasma contain hormones secreted into the bloodstream from endocrine organs like the gonads, adrenal, and pituitary along with growth factors and other substances produced locally in a variety of tissues. Steroid and peptide hormones produce most of their actions by binding to nuclear or membrane-bound receptors that vary in concentration over time and from one tissue to another, resulting in varying responsiveness to the hormonal concentration. Some steroid actions, especially those in the brain, also are dependent on the local

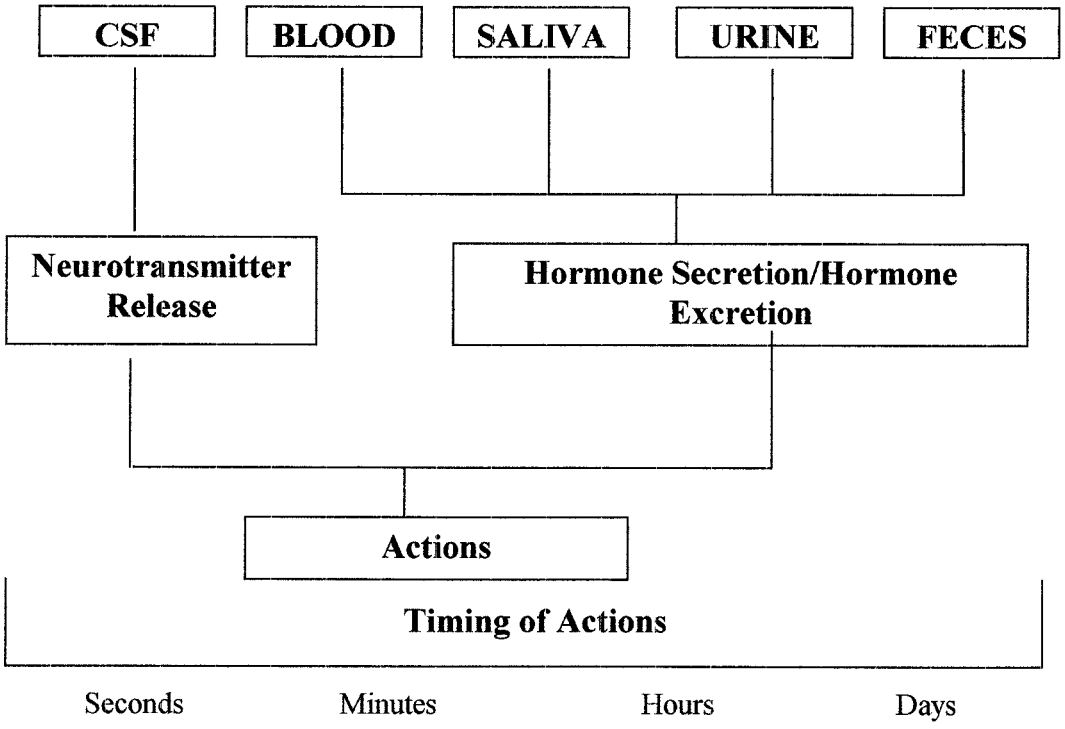

Fig. 1. Timing of endocrine release and action.

metabolism of steroids to more active forms. Therefore, circulating concentrations often provide more information about the regulation of hormone secretion and production than about hormone actions.

An additional complexity is the lag time between hormonal stimulus (binding to a receptor or other enzymatic reactions) and the response. Some steroid responses, termed early immediate actions, occur within seconds of hormonal stimulus, but effects requiring gene transcription and protein synthesis, like most effects on behavior, occur 24–48 h later, often long after the original hormone stimulus has disappeared (O'Malley et al., 1986). Hormone concentrations in urine and feces reflect the cumulative secretion and elimination of hormones over a number of hours (see Fig. 2). Urine contains soluble steroids, peptides, and some neurotransmitter metabolites along with steroids made more soluble by conjugation with glucuronic acid or sulfate in the liver, facilitating their rapid excretion (Adlercreutz et al., 1976).

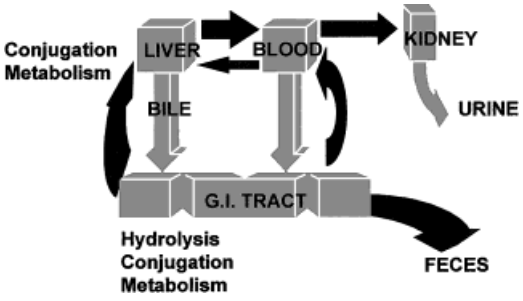

Fig. 2. Pathways of hormone excretion.

Hormones enter the gastrointestinal tract from the bloodstream and from bile. Although proteins are well metabolized by gut bacteria, steroids may be reabsorbed into the bloodstream, prolonging their residence in the body. Hormones are subsequently excreted in feces as both conjugated as well as unconjugated steroids, depending upon the species (Ziegler et al., 1996) (which may influence choice of assay methods). This array of endocrine actions suggests that

none of these measures provides a complete picture of hormone action, but rather each presents a different snapshot of endocrine systems with accompanying differences in problems of measurement and interpretation.

APPLICATIONS OF ENDOCRINE DATA

Monitoring reproductive condition

The initial impetus for the development of noninvasive endocrine methods was for analysis of ovarian cycles. To accurately assess the cyclic changes in secretion of ovarian steroids that accompany follicular maturation, ovulation, and implantation, endocrinologists must collect samples of hormone levels every few days, a procedure that is not practical in feral animals. Even in captivity, where animals can be trained to undergo blood-sampling procedures with a minimum of stress, there are limits to the frequency and duration of blood collection across different sexes and species. Frequent sampling may diminish red blood cell count, and prolonged sampling may result in the collapse of veins. Generally, blood samples are collected at most three times per week, with daily sampling during the estimated week of ovulation in the laboratory environment.

One of the earliest applications of excreted steroid analyses was in zoos to facilitate the breeding of exotic animals. Urine collected from the cage floor or from drip pans in metabolic cages provided a nondisruptive and regular means of sampling hormone concentrations. The development of steroid conjugate immunoassays that reduced time and handling costs made wide application of these assays economical (Shideler et al., 1983, 1985; Czekala et al., 1986). Comparisons to serum concentrations demonstrated that urinary steroids could provide an accurate picture of the ovarian cycle and pregnancy. The field application of urinary methods was limited initially; prior to the 1990s, only one primate study used urinary steroids to assess reproductive condition in feral, free-ranging primates. In this study, urinary steroids were used to assess the timing of mating relative to the onset of ovarian cycles and ovulation in vervet monkeys in Amboseli, Kenya (Andelman et al., 1985). Although the effort required to collect samples from identified individuals limited sampling to only a few individuals, this study fostered an appreciation of the utility of excreted steroids, culminating in the wide variety of investigations using these methods today (Tables 1, 2).

Traditionally, sexual swellings or behavioral measures have been used to estimate the timing of ovulation in the field, but these methods are inappropriate for evaluation of the patterning of sexual activity since they confound the index of physiological state with the mating tactic. Endocrine profiles permit a more reliable assessment of the reproductive significance of mating events and provide methods by which to examine the adaptive significance of mating systems. For example, copulations of Costa Rican howler monkeys were coincident with elevations in fecal estradiol but poorly associated with sexual swellings (Clarke et al., 1991). Fecal steroid profiles have shown that mating outside of the normal breeding season also is associated with ovarian activity in Brazilian muriqui monkeys, although not all copulations are restricted to the periovulatory period (Strier and Ziegler, 1994, 1997; Ziegler et al., 1997). Further, behavioral estrus, coincident with 10–15 day periovulatory elevations in fecal estradiol, as well as some exhibition of pseudoestrus elevations in fecal estradiol 1 month prior to the onset of ovarian cycles coincided with male marking and mating attempts in Verreaux's sifaka (Brockman et al., 1995; Brockman and Whitten, 1996).

Reproductive constraints

Field application of endocrine techniques also can contribute to investigations of reproductive constraints. An understanding of the reproductive constraints imposed by ecology and the social setting can provide insights into the selective pressures that have shaped the evolution of reproductive strategies and tactics. Investigations of cooperative breeding provide excellent examples of cases where proximate mechanisms have illuminated functional significance and evolutionary mechanisms (Mumme, 1997).

TABLE 2. Validations of excreted steroids as measures of ovarian function and pregnancy in species other than primates[1]

Taxa	Common name	Species	Fecal/urine steroid metabolites	Application	Reference
Aves					
Psitticinae	Cockatiel	*Nymphicus hollandicus*	E_2, T	Sex determination	Bercovitz et al., 1978; Tell and Lasley, 1991
	Domestic fowl		E_2, T	Sex determination, gonadal function	Cockrem and Rounce, 1994
Mammalia					
Artiodactyla					
Bovidae	Bighorn sheep	*Ovis candensis*	PdG	Pregnancy	Borjesson et al., 1996
	Bison	*Bison bison*	Estrogens, P_4, uE_1C, uPdG	Estrous cycle, ovulation, pregnancy	Kirkpatrick et al., 1993; Komers et al., 1994
	Moose	*Alces alces*	P_4, uPdG	Estrous cycle, pregnancy	Monfort et al., 1993
	Cow, musk ox	*Bos taurus, B. moschatus*	P_4	Estrous cycle, pregnancy	Desaulneirs et al., 1989
	Shiba goat		P_4	Estrous cycle, pregnancy	Hirata and Mori, 1995
	Okapi	*Okapi johnstoni*	PdG	Estrous cycle, pregnancy	Schwarzenberger et al., 1993
	Vicuna	*Vicuna vicuna*	P_4, 20α-DHP	Estrous cycle	Schwarzenberger et al., 1995
Perissodactyla					
Equidae	Horse	*Equus caballus, E. przewalski*	Estrogens, P_4, 2α-DHP, 20β-DHP	Estrous cycle, gestation	Kirkpatrick et al., 1990, 1991; Schwarzenberger et al., 1991, 1992
Rhinocerotidae	White rhinoceros	*Ceratotherium simon*	Progestins	Ovulation	Radcliffe et al., 1997
	White rhinoceros, black rhinoceros	*Ceratotherium simon, Diceros bicurnis*	Estrogens, progestins, 20-oxo-prenanes, urinary 20-α-DHP	Ovulation, pregnancy	Hindle et al., 1992; Schwarzenberger et al., 1996; Radcliffe et al., 1997; Berkeley et al., 1997
Proboscidea	African elephant	*Loxidona africana*	Progestins	Estrous cycle	Wasser et al., 1996
Carnivora	Maned wolf	*Chrysocyon brachyurus*	Estrogens, progestins	Estrous cycle, ovulation, pregnancy	Wasser et al., 1995

[1] Abbreviations used: E_2, estradiol; P_4, progesterone; PdG, pregnanediol glucuronide; uE_1C, urinary estrone conjugates; uPdG, urinary pregnanediol glucuronide; 20-α-DHP, 20-alpha-dihydroprogesterone; 20β-DHP, 20-beta-dihydroprogesterone; T, testosterone.

Recent studies have demonstrated that pregnancy can be monitored with fecal steroids (see Tables 1, 2). Significant elevations in fecal progesterone and estradiol were evident in captive and wild Verreaux's sifaka beginning 1 and 6 weeks, respectively, after estrus and extending into the last trimester of pregnancy (Brockman et al., 1995; Brockman and Whitten, 1996), and similar elevations were apparent in pregnant baboons in the Tana River Primate Reserve, Kenya, between 6 and 11 weeks of gestation (Stavisky et al., 1995). In baboons, elevations in fecal testosterone were apparent as early as 9 days after ovulation (Stavisky et al., 1995). In addition, elevations in fecal estradiol and progesterone extending beyond the luteal phase were

indications of pregnancy in muriqui monkeys (Strier and Ziegler, 1997). Wasser (1996) reported early pregnancy differences in conceptive cycles of baboons in Mikumi National Park, Tanzania, occurring as early as 4–5 days after the estimated day of ovulation. Conceptive cycles exhibited higher levels of fecal estrone, total estrogens, and total progestins (progesterone and pregnanalones) than nonconceptive cycles on days 4–11 of the luteal phase. These elevations, representing increases in secretion beginning only 2 days after ovulation or 1 week before implantation, were too early to be a consequence of pregnancy and must represent differences in ovarian function that mediate successful conception. The lower progestin levels observed in nonconceptive cycles are of particular interest because low luteal phase progestin levels have been linked to poor nutritional condition and a lower incidence of conception in a number of traditional, non-Western human societies (Ellison et al., 1993). In the Mikumi baboons, however, luteal progestin concentrations in conceptive cycles were lower in the wet season months of peak conception than in the dry season months, when conceptions were less frequent (Wasser, 1996). Progestin concentrations also were lower in the conceptive cycles of high-ranking females than in those of low-ranking females. Although it was argued that these differences may reflect thresholds of progestin levels for conception that vary according to the optimality of the season for conception (Wasser, 1996), reliance on sexual skin detumescence, rather than the steroid profiles, to locate ovulation is a potential confound in this study. Such individual variation in the timing of detumescence relative to ovulation reduces its reliability as an index of ovulation (Whitten and Russell, 1996). If the sexual swelling varies with stressful conditions, then the observed differences in progestin levels across rank and season might reflect differences in postovulatory days selected for hormonal sampling. Nevertheless, the findings suggest that comparison of the endocrine characteristics of fertile and infertile cycles and successful and aborted pregnancies could provide some interesting new ideas regarding the interactions between physiological adaptation and reproductive constraint coincident with individual variance in reproductive success.

Although reproductive suppression does not appear to be mediated by stress in cooperative breeders (see Fig. 4), corticosteroids appear to play a significant role in reproductive suppression in competitively breeding catarrhine primates (Herbert, 1995; Berga, 1995). Chronic stress is associated with suppression of ovarian cycles in macaques and humans (Adams et al., 1985; Loriaux and Nieman, 1990). For example, in captive long-tailed macaques, low rank is associated with castration-like suppression of estrogen secretion (Adams et al., 1985). These effects occur through suppression of the gonadotropin-releasing hormone (GNRH) pulse generator by cortisol releasing hormone (CRH) and glucocorticoid-mediated reductions in pituitary sensitivity to GNRH and gonadal sensitivity to LH (Sapolsky, 1985; Sapolsky and Krey, 1988; Loriaux and Nieman, 1990). The relationship between the adrenal and ovarian axes can be examined through the use of fecal steroid analyses. Fecal cortisol concentrations in free-ranging female baboons are significantly higher in nonpregnant reproductive states than during pregnancy (Stavisky, 1994), which suggests that animals that excrete lower concentrations of cortisol are reproductively competent.

Monitoring stress

Corticosteroids and glucocorticoids, adrenal steroids that are secreted in response to arousal and stress, also provide information regarding the physiological well-being of free-ranging animals. The first field studies of glucocorticoids in primates were carried out by darting feral male baboons in the Masai Mara Game Reserve in Kenya (Sapolsky, 1982, 1993). These investigations took advantage of the rise in serum cortisol induced by anesthesia to assess basal cortisol and stress reactivity. Comparisons of males by rank showed that high-ranking males had lower cortisol levels and more efficient responses to stress than low-ranking males but only when the hierarchy was stable.

These differences in glucocorticoid secretion were found to be less a marker of rank than a measure of individual differences in behavioral style, reflecting traits such as social acuity and coping strategies (Sapolsky and Ray, 1989; Virgin and Sapolsky, 1997). The association of measurable endocrine differences with differences in individual behavior offers a promising means of investigating the genetic basis of individual differences in reproductive success.

The darting method, however, limits the information that can be gained about the stressfulness of ongoing events and their influence on reproductive function. Noninvasive methods permit more continuous monitoring that can be used to assess day-to-day changes in cumulative stress. Excreted corticosteroids may provide a better picture of overall stress than serum corticosteroids because the excreted sample represents cumulative secretion over a number of hours. However, the pulsatile and episodic pattern of glucocorticoid secretion complicates tests of the utility of excreted steroids for measuring stress. Because excreted samples represent many hours of secretion, excreted profiles do not exactly mirror serum concentrations, and therefore day-to-day correlations between serum and excreted glucocorticoids are not as informative as correlations of ovarian steroids. Human studies have shown that urinary cortisol is correlated not only with serum free cortisol but also with the cortisol production rate (Barton et al., 1993), a finding that supports the assumption that excreted steroids provide a more cumulative index of adrenal activity. Significant correlations of fecal and serum corticosteroids have been demonstrated in rhesus macaques (Stavisky, 1994), sooty mangabeys (Whitten, 1997), and northern spotted owls (Wasser et al., 1997).

The most relevant test, however, is whether corticosteroid excretion reflects stressful states or experiences. Physical or social stressors also have been used to assess the stress responsiveness of excreted steroids. Dissociative anesthetics are well-documented stressors that induce cortisol secretion in unhabituated primates (Puri et al., 1981; Sapolsky, 1982). Threefold increases in urinary and fecal cortisol following anesthesia in captive chimpanzees demonstrated that excreted steroids were able to detect the stress of a brief intervention (Whitten et al., 1998). Increases in urinary cortisol were observed 1 day following anesthesia, whereas 2 days passed before elevations in fecal cortisol were apparent, reflecting differences in the rate of excretion of urinary and fecal steroids. Individual differences in baseline cortisol also were observed in both urine and feces, indicating that excreted steroids were useful for assessing both chronic and acute stress in chimpanzees. Handling procedures such as relocation, physical restraint, venipuncture, and surgery also have been used to document the stress responsiveness of excreted corticosteroids in captive felids (Carlstead et al., 1992), long-tailed macaques (Crockett et al., 1993), sooty mangabeys (Whitten, 1997), and northern spotted owls (Wasser et al., 1997).

The utility of fecal and urinary corticosteroids also has been assessed by their association with social rank and social interactions. Correlations with rank are probably the least satisfactory method of assessing the reliability of excreted corticosteroids because the relationship of corticosteroid secretion with rank varies with social context and behavioral style (Sapolsky, 1993). The relation of urinary and fecal corticosteroid with rank varies considerably across studies. Urinary cortisol was significantly elevated in the lowest-ranking female in a group of semi-free-ranging timber wolves and in an aggressive male whose rank was unstable (McLeod et al., 1996). However, in other cooperatively breeding carnivores, the opposite tendency was found: in mongoose females and wild dogs, urinary cortisol and fecal corticosterone, respectively, were highest in dominant animals (Creel et al., 1996). Even more variable results have been found in primates. Urinary cortisol and prolactin did not vary with rank in female Sumatran long-tailed macaques, but low-ranking males had significantly higher urinary cortisol than higher-ranking males (van Schaik et al., 1991). Preliminary data from captive long-tailed macaques suggest that the highest fecal cortisol levels were found in middle-

ranked females (Stavisky et al., 1997). Additionally, there were no differences by rank in urinary cortisol in mountain gorilla males, although cortisol was higher in immature males than in adult or developing males (Robbins and Czekala, 1997). These results suggest that additional factors play an important role in baseline stress and stress response. More consistent patterns have been observed when excreted steroids were related to social events. For example, marked elevations in urinary cortisol were observed in an immigrating male long-tailed macaque and in a mother following the kidnapping of her infant (van Schaik et al., 1991) and in a subordinate timber wolf female following a group attack (McLeod et al., 1996). These findings suggest that excreted steroids are more clearly associated with ongoing behavioral interactions than with social status.

Urinary ketones are another measure of stress that has been applied to assess nutritional condition in feral orangutans (Knott, 1996, 1997). Ketone bodies (aceotacetic acid, β-hydroxybutyric acid, and acetone) are products of excessive metabolism of fatty acids from adipose tissue in response to starvation or carbohydrate deprivation (Stein, 1987) and are a common accompaniment of weight loss in mammals (Robinson and Williamson, 1980). Ketone production by orangutans has been assessed in the field using urinanalysis strips designed for human clinical use (Knott, 1997), and this method demonstrated that ketone production was associated with a period of poor fruit production and a tenfold reduction in estimated caloric intake over a fruit-rich period (Knott, 1996). Significantly lower urinary estrone conjugate levels and the absence of mating indicated that energy imbalance had significant effects on fecundity (Knott, 1996). Such findings on nutritional status have stimulated considerable interest among wildlife biologists in field application of the urinanalysis technique. Because ketones are unstable, the strips appear an ideal method for field assessment. However, strips designed for human clinical use should be validated through comparison with more standardized assessments (i.e., radio- or enzymeimmunoassay) before applying them to less closely related species and field settings where other substances present in urine that might alter the colorometric reaction (Caraway and Watts, 1987).

Nutritional status and diet, especially dietary fiber content, can profoundly influence the urinary and fecal excretion of steroids. However, the data are not consistent across either steroids or species. Increased dietary fiber has been demonstrated to have a negative effect on circulating steroid concentrations (unconjugated and total estrogens) but a positive effect on excretion (Adlercreutz et al., 1987), but the opposite effect has been observed as well (Wasser et al., 1993). The fat/fiber ratio may account for some of this variability, as fat has a negative association with fecal excretion of estrogens (Golden et al., 1986). It is proposed that fiber influences sex hormone and bile acid metabolism through a partial interruption of enterohepatic recirculation resulting in the alteration of intestinal metabolism. However, methods to control for dietary influences on steroid excretion have been proposed (Wasser et al., 1993).

Aggression, mate competition, and testosterone

Testosterone is a hormone that traditionally has been linked to male rank and aggression, but this linkage maybe less deterministic than commonly depicted (Bercovitch, 1993; Sapolsky, 1997; Whitten, in press). Early endocrine studies concentrated on the correlation between testosterone and male rank (Rose et al., 1978) in hopes of identifying the traits conferring high rank. It soon became evident, however, that many of the behavioral and endocrine characteristics of dominant individuals were consequences rather than causes of social position, reflecting the variable and complex relationship of testosterone to aggression. Whereas testosterone is very responsive to social context, rising following intermale contests and falling in response to defeat (Clarke et al., 1986), normal elevations in testosterone may facilitate continued hostilities but do not actually cause aggression or individual differences in expression of aggression. Individual differences in hormonal responses to stress are associated with behavioral differ-

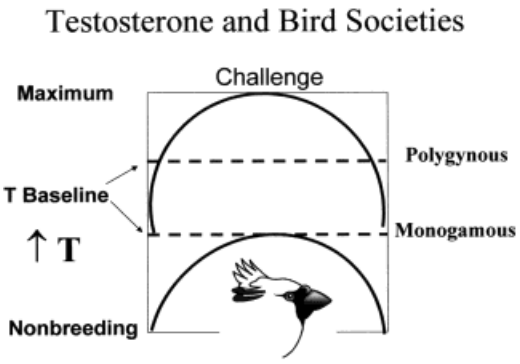

Fig. 3. Wingfield's hypothesis for the evolution of androgen-mediated mating competition in birds (Wingfield et al., 1990).

ences such as the ability to choose winning disputes and to discriminate between threatening and neutral social interactions. Excessive aggression, reactivity, and dispersal tendencies also have been linked to CSF concentrations of monoamine metabolites (Higley et al., 1991, 1992; Kaplan et al., 1995), stable individual traits that appear to have a strong genetic component (Higley et al., 1993).

Wingfield et al. (1990) and Wingfield (1994) attempted to place these results within an evolutionary framework. The challenge hypothesis argues that testosterone does not influence all aspects of aggression but rather specifically regulates mate-guarding and other aspects of reproductive aggression. Inhibitory effects of testosterone on parental behavior provide a trade-off with the benefits of mate defense. This hypothesis has led to the discovery of complex differences in testosterone across bird species. Studies of wild birds have shown that polygynous birds have higher baseline testosterone levels than monogamous birds and that testosterone treatment induces polygyny in monogamous birds (Fig. 3). However, monogamous birds retain the capacity to respond to reproductive challenges with elevations in testosterone secretion and are in fact more responsive to intrusions than polygynous birds.

A comparison of published serum testosterone means across species suggests that this pattern also might apply to primates: testosterone levels were found to be significantly higher in cercopithecoid species living in multimale groups where intermale contests would be more constant than in unimale species where contests for access to females would be more intermittent (Whitten, in press). These findings require a more comprehensive validation but suggest that it may be productive to explore the links between testosterone and reproductive aggression in primates.

The relation of androgen secretion to reproductive aggression in primates is further supported by the fact that testosterone is closely associated with rank in species in which a clear alpha male has relatively exclusive access to group females (Whitten, in press). The most extreme example of the alpha-male phenomenon is the mandrill in which markedly higher testosterone levels are associated with group-living, exclusive paternity, and more marked development of secondary sexual characteristics (Wickings and Dixson, 1992; Dixson et al., 1993; Wickings et al., 1993). Individual differences in androgen secretion and physical development are apparent well before puberty, and these differences appear to be socially based (Bercovitch, 1993; Mann et al., 1998).

Noninvasive methods provide new opportunities to examine the relation of androgens to reproductive competition. Excreted testosterone may provide a more integrative profile of androgen reactivity over a number of hours, avoiding some of the problems of measurement and interpretation presented by the minute-to-minute variability of serum testosterone. Field studies have shown that male androgens can be monitored noninvasively in free-ranging populations of primates and other mammals (see Table 3). Most analyses of urinary testosterone have not found consistent associations with rank. Testosterone was unrelated to rank in long-tailed macaques (van Schaik et al., 1991) and mountain gorillas (Robbins and Czekala, 1997), although it was related to dominance rank and breeding status in both male and female naked mole rats (Faulkes and Abbott, 1997). Analyses of fecal testosterone have found associations with male rank in mantled howling monkeys (Zucker et al., 1996) and Verreaux's sifaka (Brockman et

TABLE 3. Applications of excreted steroids in the assessment of stress, status, and aggression[1]

Taxa	Common name	Species	Hormone/ metabolite	Application	Reference
Aves	Spotted owl	*Strix occidentalis*	fC	ACTH response, habitat disturbance	Wasser et al., 1997
Mammalia					
Artiodactyla					
Bovidae	Bighorn sheep	*Ovis canadensis*	fF, uF	ACTH response	Miller et al., 1991
Perissodactyla	Elephant	*Loxodonta africana, Elephas maximus*	uF	ACTH response	Brown et al., 1995
Proboscidea					
Carnivora					
Viverridae	Dwarf mongoose	*Helogale paryula*	uF	Rank effects	Creel et al., 1996
Felidae	Domestic cat, leopard cat, cougar, Geoffrey's cat	*Felis catus, F. bengalensis, F. concolor, F. geoffreyi*	uF	ACTH response	Carlstead et al., 1992
Canidae	Timber wolf	*Canis lupus*	uF	Effects of aggression and rank	McLeod et al., 1996
	African wild dog	*Lycaon pictus*	fC	Stress of rank	Creel et al., 1996
Primates					
Indriidae	Verreaux's sifaka	*Propithecus verreauxi*	fT	Male aggression, age, and rank	Brockman et al., 1998
Callitrichidae	Cotton top tamarin	*Saguinus oedipus*	uF	Reproductive and social group effects	Ziegler et al., 1995
	Black howler	*Alouatta palliata*	fT	Age and rank effects	Zucker et al., 1996
Cercopithecinae	Longtail macaque	*Macaca fascicularis*	uF, uT, uPRL, fF	Age, rank, and reproductive status effects, well-being	van Schaik et al., 1991; Crockett et al., 1993; Stavisky et al., 1997
	Rhesus macaque	*Macaca mulatta*	fF	Relation to cycle	Stavisky, 1994
	Baboon	*Papio cyncephalus*	fT, fF	Relation to conception	Stavisky, 1994
	Sooty mangabey	*Cercocebus torquatus*	fF, fT	Sampling effects, amenorrhea	Whitten and Russell, 1996; Whitten, 1997
Hominidae	Chimpanzee	*Pan troglodytes*	fF, uF	Baseline and anesthesia stress	Whitten et al., 1998
	W. lowland gorilla, mountain gorilla	*Gorilla gorilla gorilla, G.g. beringel*	uF, uT	Diurnal pattern, puberty, rank effects	Czekala et al., 1994; Robbins and Czekala, 1997
	Orangutan	*Pongo pygmaeus*	uK	Nutritional stress	Knott, 1997

[1] Abbreviations used: fC, fecal corticosteroid; fF, fecal cortisol; fT, fecal testosterone; uF, urinary cortisol; uK, urinary ketones; uPRL, urinary prolactin; uT, urinary testosterone.

al., 1998). Zucker et al. (1996) found that fecal testosterone was positively related to rank in mantled howling monkey groups, but the consistency of the relationship varied across years and social groups. In sifaka, fecal testosterone baselines vary with male rank and age. Elevations in fecal testosterone were associated with male transfer, intergroup aggression, and extragroup mating, and reductions were associated with rank reversal and peripheralization (Brockman et al., 1998). At this point, there are too few studies to judge whether these differences in outcome reflect choice of species, group dynamics, or differences in the information conveyed by urinary and fecal steroids.

SAMPLING AND ASSAY METHODS

Sample collection

A variety of samples can be collected from feral animals. All of these samples can pro-

vide physiological information; however, species, habitat, and research questions often require different data. Below is a description of each sample type, utility, advantages, and disadvantages.

Blood

Blood sampling is the most traditional medium for endocrine analysis. Serum and plasma hormone concentrations are well established in the literature for a variety of species. The disadvantage of collecting blood samples, however, is that feral animals must be trapped or darted to obtain blood samples, putting them at potential risk and altering behavioral or endocrine states. The latter effect is not necessarily an obstacle and in fact has been put to use in field studies of baboons where cortisol responses to immobilization have been used to assess individual differences in reactivity (Sapolsky, 1982).

Cerebral spinal fluid

CSF samples can be collected in the field but require darting of subjects and sterile preparation of the sampling site (Phillips-Conroy, personal communication). Information about neurotransmitter levels can be obtained from a relatively simple sampling procedure. However, like blood, CSF samples provide only a single snapshot in time. Noninvasive methods, on the contrary, permit an analysis of the interactions between social, environmental, and endocrine experiences.

Saliva

Saliva is an excellent source of both steroid and peptide hormones. Sensitive assays permit both ovarian cycle monitoring (Worthman et al., 1990) and measurements of stress hormone activity (Fuchs et al., 1997). Saliva collection can be a noninvasive procedure with habituated or laboratory-housed animals, eliminating the potentially confounding effects of stress response to sampling method. However, saliva collection may prove to be too challenging in the field environment with feral animals or at least for most species.

Urine

Excreted samples provide an alternative means of assessing hormone concentrations in feral animals. Urine aliquots of up to 15 ml can be collected from a tarp (Knott, 1997) or bowl (Andelman et al., 1985) placed under the animal's sleeping or resting site, and smaller amounts (less than 1 ml) often can be collected from vegetation (Knott, 1997; Robbins and Czekala, 1997). The collection of large samples is likely to depend on habituation, the location and height of resting and urination sites, and travel rate. Collection of urine-soaked snow is a novel method that was found useful for the collection of samples from feral horses (Kirkpatrick et al., 1990) and Canadian timber wolves (McLeod et al., 1996), but in most climates urine from terrestrial species may soak into the ground before it can be collected. The utility of collecting the smaller amounts that might be obtained from vegetation will depend on the number of hormones to be assayed, the amount of sample required per test, and the reliability with which individual samples can be identified. Urine samples have been successfully collected from free-ranging vervet monkeys (Andelman et al., 1985), long-tailed macaques (van Schaik et al., 1991), orangutans (Knott, 1997), gorillas (Robbins and Czekala, 1997), and chimpanzees (Wrangham, personal communication). However, urine sample collection can be confounded by a wet or rainy field site, either diluting samples or making them indistinguishable in the environment.

Feces

Collection of fecal samples is more feasible for relatively terrestrial animals for the assessment of steroids or DNA since the samples are more readily identified from a distance and relocated. Individual samples can be marked for later location by markers (Komers et al., 1994) or by dyes fed to animals in food or administered in concert with capture and anesthesia (Stavisky et al., 1997). It is rarely necessary to collect samples larger than 1 g for assay since steroids tend to be present at high concentrations in feces. Like urine collection, the collection of fecal

samples is noninvasive; thus, the sampling method does not intrude on either behavioral or endocrine states. However, care must be taken to prevent misidentification of samples, for both urine and fecal sample collection, when study animals sleep or group closely together.

Any bodily fluid is potentially infectious and should be handled with care. Researchers should wear disposable latex gloves when collecting or handling samples and should use goggles and a disposable mask to protect from aerosols if the material is mixed, homogenized, pipeted, or centrifuged. Liquid samples should be stored in unbreakable, solvent-resistant containers with leak-proof, screw-top lids. An import permit from the Centers For Disease Control and Prevention (Office of Health and Safety) is required to pass through United States customs as well as permits for the collection and removal of the samples from the country where the study is being undertaken.

Preservation methods

Steroid stability. Serum steroids are stable for up to 72 h at room temperature, for at least a week at 4°C, and for many years when stored at -20°C in airtight, nonbreakable containers (Grant and Beastall, 1983; Bolelli et al., 1995), but nonpolar steroids like progesterone and androstenedione are very unstable unless protein is added (Chattoraj and Watts, 1987). In long-term cryopreservation (e.g., 3 or more years), progesterone may be absorbed into the walls of some plastic containers, and sex steroids also may dissociate from their binding globulin with time, increasing the apparent free fraction (Bolelli et al., 1995).

Oxidation and bacterial metabolism can alter steroid concentrations in unpreserved samples. The problem of bacterial metabolism is particularly marked in excreted samples where gastrointestinal bacteria are abundant in the sample. Wasser et al. (1988) showed that changes in fecal estradiol (fE_2) and progesterone (fP_4) concentrations occurred very rapidly (within 6 h) in unpreserved samples kept at room temperature. Urinary steroids are more stable, with no changes in concentrations for at least 24 h at room temperature and at least 72 hr at 4°C (Grant and Beastall, 1983). Conjugates of estrone and pregnanediol decline in concentration within 1–2 weeks at ambient temperatures (Kesner et al., 1995), although urinary cortisol may be stable for as long as 8 weeks (Brown et al., 1995).

Field preservation techniques. A variety of preservation methods have been used in the field. Freezing is a very reliable method for steroid preservation if a freezer or liquid nitrogen canister is available, and this method has been used to preserve serum and urine samples collected from primates in East Africa (Sapolsky, 1982; Andelman et al., 1985; Phillips-Conroy et al., 1992; Czekala et al., 1994) and Indonesia (van Schaik et al., 1991). Researchers may have difficulty in shipping these samples, however, since airlines frequently restrict dry ice or liquid nitrogen transportation. Moreover, freezing alone does not adequately preserve fecal DNA, reducing the appeal of this method for those interested in putting samples to multiple uses. Freezing in 95% ethanol does preserve fecal DNA, however, and this method has been used by several primatologists to preserve fecal steroids (Wasser et al., 1991; Strier and Ziegler, 1994, 1997).

Aqueous ethanol, sodium azide, and acids are the substances commonly used to preserve steroids at room temperature (Young and Bermes, 1987; Brown et al., 1995). Serum steroids are stable for short periods of time with the addition of sodium azide and can be preserved for at least 3 weeks when spotted onto filter paper (Worthman and Stallings, 1997). Preservatives have negligible effect on the warm-temperature stability of urinary estrone and pregnanediol conjugates (Kesner et al., 1995), but urinary cortisol retains 89% of its activity for 12 weeks in 1O% ethanol or 0.1% sodium azide (Brown et al., 1995). Preservation in 95% ethanol at room temperature is the method most commonly used to preserve fecal DNA, and a number of researchers have used alcohol solutions to preserve fecal samples for steroid analysis. Wasser et al. (1988) showed that addition of ethanol (8 ml/gm) stabilized fE_2 and fP_4 concentrations for 21 h but did not provide data on room-tempera-

ture stability over longer periods of time. When solvent-based preservation methods are used, care must be taken to account for the effects of the solvent on steroid partitioning and assay reliability. For example, preservatives used to preserve stool samples for parasitology are inappropriate for steroid analysis because some components cross-react with steroid antibodies (Whitten, unpublished data). We have found that the majority of steroid is extracted into the solvent when feces are suspended for prolonged periods in 90% ethanol at ambient temperatures (unpublished data). Unless samples are weighed at the time of collection, this partitioning may make it difficult to normalize steroid concentrations by fecal weight, the usual way of expressing these values. However, samples may be lyophilized to normalize steroid concentration by fecal weight. Other problems with the use of ethanol include its flammability and its potential for evaporation. Isopropyl alcohol has less evaporative potential and has been used to preserve ovarian steroids and testosterone in stool samples collected from Costa Rican mantled howler monkeys (Zucker et al., 1994, 1995, 1996).

Drying fecal samples in field sites where organic solvents and electricity are not available (Brockman and Whitten, 1996) is a useful preservation technique. The sample is packaged in foil, pressed flat, and oven-dried 55–85°C for 2–3 h. Laboratory tests demonstrated that this method resulted in high recovery (76–86%) and 100% stability of estradiol and testosterone over a 3 week period. Recovery (43–50%) and stability (75%) were lower for progesterone. Regardless of the differences in recovery, field collections indicate that all three of the steroids are present in measurable amounts and yield interpretable profiles for 3 years or more after collection (Brockman and Whitten, 1996).

Several field-extraction methods have been devised. These methods involve a preliminary extraction in the field, having the advantage of eliminating the need to ship stool samples or organic solvents. One method is based on the use of filter and extraction cartridges that can be used with disposable syringes (Stavisky et al., 1995). This method requires a scale and the availability of organic solvents. A small sample of stool (0.1 g) is dissolved in 2 ml ethanol:acetone (8:2) and passed through a 0.2 micron nylon or Teflon (PTFE) filter. The filtered extract is diluted with an equal volume of water and loaded onto a C 18 solid phase extraction cartridge (prepared according to the manufacturer's instructions: Millipore, Milford, MA; Waters Chromatography) and washed with 1 ml of a 0.1% sodium azide solution. Gonadal steroids such as estradiol, estrone, estriol, and testosterone are stable for at least 8 weeks using this method. Field tests in Kenya demonstrated recoveries of near 100% for estradiol and 73% for progesterone in comparison to frozen samples. The lower recovery for progesterone may reflect the use of plastic containers and syringes which may absorb progesterone. One obstacle to applying this method in the field is that solubilization in organic solvent may be incomplete without a motorized homogenizer, and, as a result, particulate matter may clog the cartridges, a problem that could be solved by spinning the sample in a manual centrifuge, by allowing the extract to settle for several hours, or by substituting water for the organic solvent.

An alternative extraction method dissolves fecal steroids in an aqueous solution and applies the solution to filter paper, a method also used to preserve urine samples. Urine samples are applied to filter paper by soaking in a urine sample (Shideler et al., 1995), a method which requires standardization of soaking time and of the liquid absorbed per area of paper, or by applying replicate measured aliquots using a micropipet (Knott, 1997). The same method has been used to preserve fecal samples solubilized at a 1:50 dilution in distilled water (Shideler et al., 1995). Filter papers used include Whatman (Clifton, NJ) #1 (Shideler et al., 1995) and Schleicher & Schuell (Keene, NH) #16110 (Knott, 1997). After application of the sample, the filter paper is dried and stored along with a desiccant like silica gel. Although only a few profiles have been tested against serum using these methods (Knott, 1997: n = l; Shideler et al., 1995: n = 6), the results suggest that filter-paper preservation provides interpretable profiles (Shideler

et al., 1995) with estrone-conjugate concentrations that parallel concentrations in matched frozen samples (Knott, 1997). After 1 month of storage, filter-paper concentrations of fecal estrone conjugates and pregnanediol-3-glucuronide were well correlated with serum estradiol and progesterone concentrations in conceptive cycles (Shideler et al., 1995). Urinary estrone conjugate concentrations measured using the filter-paper method were identical to initial concentrations over a 1 year period and were 10–50% less, but with a similar profile, after 5 years (Shideler et al., 1995).

An additional extraction method yielding high steroid and steroid metabolite recoveries utilizes a small amount of lyophilized fecal sample (0.1 g dry weight) extracted in 5 ml distilled water:ethanol (50:50), followed by vortexing and centrifugation, respectively (Strier and Ziegler, 1997). Following centrifugation, the fecal pellet is discarded, and two aliquots are removed for diethylether (estrogens) or petroleum (progesterone) extractions (Strier and Ziegler, 1997). Accuracy and recovery assessments suggest that this method is highly reliable.

Thus, a variety of methods are available for preserving hormonal samples in field settings. As yet there has been no comprehensive comparison of these methods and their efficacy, but all have been applied with some degree of success in field settings. Because the amount of sample required to measure a hormone varies considerably across assays, the protocol for preserving samples should be derived in consultation with the endocrinologist who will carry out the hormone assays.

Sampling frequency

For analysis of ovarian cycles, daily or near-daily samples are ideal, and thrice-weekly samples will yield sufficient information to determine whether there is follicular development or formation of a corpus luteum for some species. Daily samples would be required for determining the timing of ovulation (Whitten and Russell, 1996); they are not required to assess baseline stress levels using cortisol or corticosterone. Clinical studies suggest that at least 18 samples per individual are required to reliably estimate mean salivary cortisol (Coste et al., 1994).

Individual differences in testosterone also can be detected using less frequent sampling. Zucker et al. (1996) found that one to three samples per month provided consistent estimates of fecal testosterone (fT) levels for individual mantled howler monkey males that were stable over a 3 year period. Weekly fecal samples also were sufficient to detect rank- and aggression-related differences in testosterone levels among male sifaka (Brockman et al., 1998). More frequent sampling of adrenal and gonadal steroids would be required to assess day to-day responses to behavioral events, however.

On a population level, useful cross-sectional data might be obtained using only one or a few samples per individual. For example, in human studies, assays of progesterone in single blood samples (repeated over multiple cycles) have been used to assess the frequency of ovulatory cycles or pregnancy (Leslie and Fry, 1989). These approaches may be more problematic with excreted steroids, however, and laboratory analyses will be required to assess the degree of variability in hormone levels within reproductive phases in order to interpret these data accurately (Sobolik et al., 1996). Samples from animals that use distinct areas to deposit feces to mark their territory might be used to census the number of reproductive individuals of each sex; genetic markers from the Y chromosome could be used in conjunction with sex steroids to assess the sex and reproductive status of individuals in the population (Kohn and Wayne, 1997). Steroid stability could be an important obstacle, however, and requires the prior determination of the reliability of aged samples for assessing the sex of the depositor. A ratio of estradiol to testosterone has proven useful for identifying sex and menstrual-cycle stage in ancient and modem human samples (Sobolik et al., 1996) and might be useful in population censuses.

The number of individuals sampled, however, is dependent upon a number of factors. The questions that are being addressed by steroid analyses, the particular hormones of interest, and the species being studied all influence data interpretation. Statistical

power calculations may be applied to determine the appropriate number of study animals.

Time lags to excretion

Timing of the collection of samples with respect to behavioral or physiological events varies with the type of sample. Whereas serum hormones reflect endocrine responses and states at the moment of sampling, excreted hormones reflect events occurring hours to days before. Urinary steroids are generally excreted more rapidly than fecal steroids, with peak excretion within 4–8 h and completed excretion within 24 h (Ziegler et al., 1989; Crockett et al., 1993; Wasser et al., 1994). In contrast, fecal steroids are excreted at intervals of 0.3–3 days after secretion (Perez et al., 1988; Ziegler et al., 1989; Heistermann et al., 1993; Shideler et al., 1993; Wasser et al., 1994), reflecting the longer duration of gastrointestinal transit, which ranges from a few hours to several days. Characterization of lag times is important when attempting to determine the timing of physiological events like ovulation or to test the relation of hormone levels to behavioral events and states. For example, the prolonged lag times to fecal excretion may favor the use of fecal samples to assay hormonal baselines during interventive procedures; cortisol responses to a stressful procedure would not be seen in feces until 2 to 3 days after the procedure, whereas changes in serum cortisol would be evident within 30 min. On the other hand, prolonged lag times combined with the recirculation of steroids from the gut are likely to make fecal steroids less-sensitive indices of the stress of a single event.

Timing of sample collection

Careful attention should be given to the timing of sample collection. If possible, samples should be collected at a regular time each day. In many free-ranging primate species, it may be easier and more reliable to collect stool or urine samples in the morning, when animals are still immobile in their sleeping trees, than at other times of the day. However, circadian rhythms of hormone secretion should also be taken into account. Early morning is the most ideal time for collection of urine samples for analysis of ovarian steroids since urine samples are more concentrated as a result of overnight inactivity and represent a larger proportion of the day's excretion (Collins et al., 1979). Most studies that have assessed ovarian cycles of primates using stool samples also have relied primarily on morning samples, although there is no evidence that morning stool samples are better than samples taken at other times (Brockman and Whitten, 1996). Because sample water content does not appear to be as much a confound in measuring fecal steroid concentrations as it is in urine samples (Shideler et al., 1993), the overnight period of inactivity may not have a significant influence on fecal steroid concentration. However, circadian patterns of hormone secretion can influence both fecal and urinary steroid concentrations. Estrogens do not appear to have a constant circadian rhythm in serum or feces (Norjavaara et al., 1996; Hess et al., 1981), but cortisol, testosterone, and progesterone all exhibit marked variation in secretion over the course of the day. Serum cortisol peaks before the onset of activity and declines over the course of the day to a late afternoon–early evening trough (Krieger, 1978; McIntosh et al., 1981; Coe and Levine, 1995). Urinary cortisol exhibits a parallel pattern, reaching a nadir around midnight (Eckert et al, 1991; Czekala et al., 1994). Detecting hypercortisolemia is easier in the nadir than in the hours of peak secretion or excretion where within-individual variation is more marked (Krieger et al., 1971; Contreras et al., 1986; Zis et al., 1990; Czekala et al., 1994), but nighttime collections are likely to be impractical for studies of most free-ranging animals. The longer lag time to excretion of fecal cortisol may provide a more opportune timing of excretion (e.g., morning or afternoon) for sampling the nocturnal nadir in secretion.

Although serum testosterone concentrations exhibit a similar rhythm to that of cortisol in hominoids, a rather different pattern is apparent in Old World monkey males, where testosterone peaks around midnight and reaches a nadir in the early-morning hours (Perachio et al., 1977; Beattie and Bullock, 1978; Plant, 1981). Female and

castrated male cercopithecoids, on the other hand, exhibit the hominoid pattern, suggesting that this pattern reflects adrenal rhythms (Perachio et al., 1977). The utility of nocturnal vs. diurnal testosterone for assessing male differences in gonadal function has not been addressed; for practical reasons, most studies have sampled males in the daytime hours. The longer lag time to fecal excretion, however, makes it possible to examine this nocturnal pattern of secretion since daytime stool samples in some species may reflect steroid secretion during the late-night hours.

Extraction methods

Most laboratories have homogenized fecal samples in methanol or ethanol in combination with water or acetone for a preliminary steroid extraction (Adlercreutz and Jarvenpaap, 1982; Risler et al., 1987; Shideler et al., 1993; Stavisky et al., 1995; Strier and Ziegler, 1997; Ziegler et al., 1997). The extract is often further purified using ether (Heistermann et al., 1993; Strier and Ziegler, 1997) or dichloromethane (Wasser et al., 1991), solid-phase extraction (Stavisky, 1994; Stavisky et al., 1995), celite chromatography (Ziegler et al., 1996), diethyl ether, petroleum (Strier and Ziegler, 1997), or a series of solvent and chromatographic steps (Risler et al., 1987). Solvolysis may be used to hydrolyze conjugated steroids (Ziegler et al., 1996). Some investigators have developed single-step procedures to speed the extraction process, using overnight shaking in phosphate buffer with 20% methanol (Shideler et al., 1993) or boiling in aqueous ethanol (Wasser et al., 1994). One concern with the elimination of purification steps would be the possible retention of substances that might interfere with steroid immunoassay, reducing accuracy or precision. Boiling may alter steroid composition or cause degradation of more labile steroids like glucocorticoids. Therefore, these methods should be tested in conjunction with the intended radioimmunoassays.

Assay specificity

Immunoassays have achieved a high degree of specificity in the measurement of serum hormones. Excreted samples, however, contain a number of hormonal metabolites and other substances not present in serum that may interfere with the accuracy and reliability of hormone assays. Therefore, any immunoassay should be validated for the species and sample of interest prior to the analysis of samples. Investigators have taken varying approaches to the issue of specificity in the assay of excreted steroids. Endocrinologists have generally opted for highly specific assays for the measurement of urinary steroids, but a variety of approaches have been taken in the assay of fecal steroids. Some investigators have chosen assays that are highly specific to the hormone of interest, whereas others have applied less specific assays that react with a number of its common metabolites. Less specific assays may more fully measure the metabolites of a given hormone but also may interact with hormones and metabolites derived from other endocrine axes. Low specificity also may make it more difficult to interpret fecal steroid profiles in the context of data based on serum steroids.

One the other hand, assay specificity may be constrained by patterns of steroid metabolism. Infusions of radiolabeled steroids in primates indicate that estradiol is excreted primarily as estradiol and estrone or its conjugates, whereas progesterone is often metabolized into a number of progestin metabolites (Ziegler et al., 1989; Shideler et al., 1993; Wasser et al., 1994). For example, pregnanelones and pregnanediols were found to be the primary metabolites of progesterone in baboons (Wasser et al., 1994). Consequently, progestin antibodies often react with a number of fecal metabolites, whereas estrogen assays are often relatively specific when applied to fecal extracts (Heistermann et al., 1993; Shideler et al., 1993). In a number of ungulate species, a large number of progestins are present in feces, primarily pregnanes and pregnanediones, and group-specific assays to the most common type of pregnanes have proven most useful (Schwarzenberger et al., 1996; Palme et al., 1997).

The immunoassay kits used to detect excreted steroids were developed for use in human serum, plasma, or urine; thus, the kits may not have been tested for cross-reactivity with metabolites that may be pre-

sent only in urine or fecal samples. High performance liquid chromatography (HPLC) can be used to identify and test the immunoreactivity of the major steroid and steroid metabolites present in extracts for the determination of antisera accuracy and cross-reactivity of different radioimmunoassay kits. Steroid separation and identification is achieved through injecting aliquots of extract under high pressure through a column equipped with a fraction collector. Retention times of the fractions are compared with authentic steroid standards for the identification of individual steroid components. Identification of steroid and steroid metabolites is also achieved through a comparison of absorption spectra collected for each peak during chromatography to spectra of authentic standards. A range of chromatographic procedures is available, including estrogen programs, androgen programs, and adrenal programs, which provide information regarding the majority of excreted steroids and steroid metabolites. Assay kits can be employed to test the different fractions collected during the HPLC procedure.

Of course, the ultimate test of the efficacy of an assay is its predictive value. A variety of estrogen (estradiol, estrone, estriol, estrone conjugate, total estrogens) and progestin (pregnanediol glucuronide, progesterone, 20-alpha-hydroxyprogesterone) antibodies have been tested in fecal extracts, and most have provided good correlations with serum or urinary profiles of the ovarian cycle. Less is known about the metabolism of adrenal and testicular steroids, but antibodies to cortisol, corticosterone, and testosterone have yielded good correlations with urinary steroids or behavioral events. Shideler et al. (1993) found that a more specific pregnanediol glucuronide assay did not provide a better correlation with serum progesterone than a less specific assay, suggesting that there is no single metabolite that must be measured in order to obtain informative results. However, it is important to note that the different steroid and steroid metabolites present in urine and feces are species-specific, and the levels that are excreted frequently represent a fraction of circulating hormones. Thus, it is important to validate extraction, preservation, stability, and assay techniques for each species studied.

CONCLUSIONS

Applications and validations of excreted hormone techniques have increased exponentially over the last 10 years. The development of these methods is a response to the recognition by researchers that behavioral studies may be incomplete without an associated examination of underlying physiological mechanisms. The recent studies that have combined hormone-behavior measurements have provided data that provide new information into the species, population, and individual differences in the mating system, dominance relationships, and dispersal patterns of primates (Sapolsky 1982, 1989; Virgin and Sapolsky, 1997; Kaplan et al., 1995) as well as reproductive condition (Clarke et al., 1991; Stavisky et al., 1995; Heistermann et al., 1996; Brockman and Whitten, 1997; Strier and Ziegler, 1997) The accumulated data support the use of excreted hormones as measures as specific as ovarian function, pregnancy, aggression, and stress, providing a springboard for investigation of hormone-behavior interactions in field settings. In fact, the information provided by the assessment of excreted steroids may provide the only information regarding reproductive condition for some species (Ziegler et al., 1997). These discoveries promise to provide approaches for the examination of the costs and benefits of behavioral strategies and their endocrine regulation, expanding our understanding of the evolution of social behavior (Fig. 4).

The noninvasive nature of the methods is especially important. The traditional practices for the collection of physiological samples, which relied upon capture, restraint, and venipuncture, had the potential to markedly influence the hormones that were being assessed. The collection of excreted hormones provides more accurate representations of endocrine processes.

There are currently a number of methods available for the collection of physiological samples in the field. Each of the methods has demonstrated reliability, validity, and practicality. Each technique is well suited for a particular set of scientific questions or

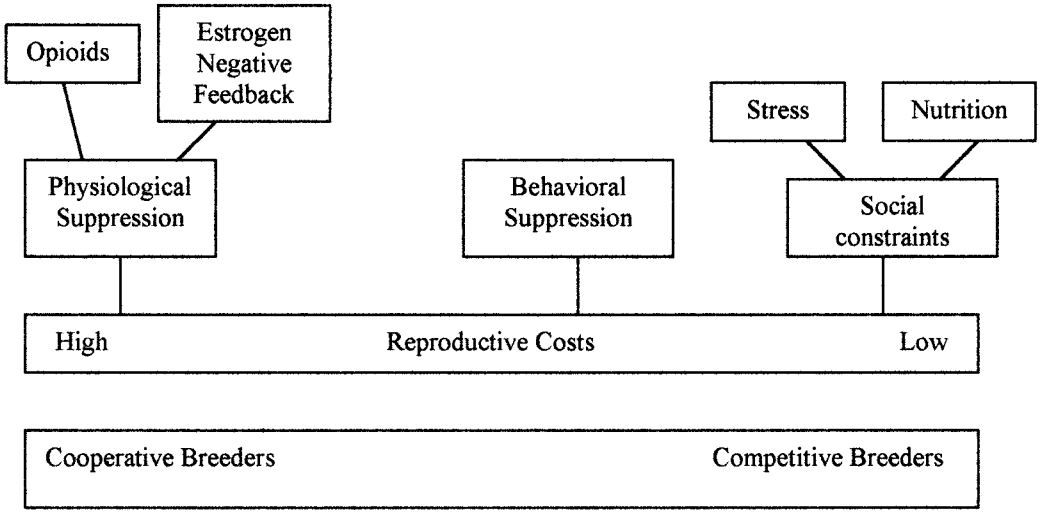

Fig. 4. Mechanisms mediating reproductive skew in cooperatively and competitively breeding mammals.

species. The broad range of available techniques provides researchers with opportunities to gather physiological data from an assortment of species living in a variety of environments. Noninvasive collection of endocrine data has expanded the opportunities for the study of hormone-behavior interactions in free-ranging animal species.

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